FEBS Letters 589 (2015) 3302–3308

journal homepage: www.FEBSLetters.org

MicroRNA-145 regulates osteoblastic differentiation by targeting the transcription factor Cbfb

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ARTICLE INFO

Article history:
Received 13 June 2015
Revised 4 August 2015
Accepted 24 September 2015
Available online 9 October 2015

Edited by Zhijie Chang

Keywords:
MicroRNA
Osteoblast
Core binding factor beta

ABSTRACT

Osteoblastic differentiation is regulated by various factors, including hormones and transcription factors. Runt-related transcription factor 2 (Runx2) is an essential player in osteoblastogenesis and transactivates its molecular target by creating a protein complex with its hetero-dimeric partner core binding factor beta (Cbfb). However, the molecular regulation of Cbfb expression remains unknown. Here, we identified miR-145 as a crucial regulator of Cbfb expression. The expression of miR-145 increased during osteoblastogenesis, indicating that miR-145 works as an inhibitor of osteoblastogenesis. Stable expression of miR-145 decreased endogenous Cbfb expression and inhibited osteoblastogenesis, in cooperation with miR-34c. Furthermore, miR-145 decreased bone regeneration in vivo. Our results indicate that miR-145 physiologically regulates osteoblast differentiation and bone formation via Cbfb expression by forming a regulatory microRNA network.

1. Introduction

Skeletal development and bone regeneration depend on the properties of osteoblasts derived from mesenchymal stem cells (MSCs) [1–5]. Bone is routinely remodeled throughout life, requiring the coordinated expression of many genes in response to many physiological signals. Osteoblastic differentiation from MSCs is regulated by multiple signaling pathways that are activated ligands such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and Wnts, which directly activate runt-related transcription factor 2 (Runx2) [6–8]. Runx2 is an essential transcription factor for osteoblastic differentiation and Runx2-knockout (KO) mice die at birth due to the absence of osteoblasts [9–12]. To activate the transcription of its target genes, Runx2 requires core binding factor beta (Cbfb), a co-transcription factor that enhances the DNA binding of Runx2, although it does not bind DNA by itself [13]. Indeed, Cbfb mutant mice develop skeletal dysplasia and die at birth, displaying a phenotype reminiscent of Runx2-KO mice [14–17].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate the expression of the target protein by inhibiting the translation or accelerating the degradation of the transcript through binding to the 3′-UTR of those target genes [18]. MiRNAs are important regulators in various developmental, physiological and pathological conditions [19–21]. Previous reports suggested that miRNAs are also intimately involved in the osteoblast differentiation. For instance, miR-206 inhibits osteoblast differentiation by suppressing connexin43 [22]. MiR-29 and -218 promote osteoblast differentiation by modulating Wnt signaling [23–25]. MiR-26a and -100 functionally inhibit osteoblast differentiation by targeting BMP signaling [26,27]. Moreover, numerous studies have revealed that multiple miRNAs including miR-204, -133, -23a, -30c, -34c, -133a, -135a, -137, -204, -205, -217, and -338 inhibit osteoblast differentiation by targeting Runx2 [28–31]. However,

Author contributions: T.F. conducted most of the experiments. H.O., S.S., A.H., W.B. and H.I. conducted in vitro experiments. Y.A. conducted mice analysis. A.O. discussed the project. T.F. and S.T. wrote the manuscript text. S.T. designed and supervised the project.

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http://dx.doi.org/10.1016/j.febslet.2015.09.024
0014-5793/

the miRNAs that regulate Cbfb, an essential partner for Runx2, remain unknown.

In this study, we identified miR-145 as an miRNA that targets Cbfb. We also demonstrated that miR-145 inhibits osteoblastic differentiation in cooperation with miR-34c, which targets Runx2. We propose that osteoblast differentiation is regulated by an miRNA network system targeting the Runx2-Cbfb transcription factor complex rather than a single microRNA targeting a single protein.

2. Materials and methods

2.1. Reagents and plasmid

Mimic miR-145, miR-34c and control miRNA were purchased from Dharmacon. A genomic fragment of miR-145 and a cDNA fragment of Cbfb were amplified by PCR and cloned into pcDNA3.

2.2. Cell culture and transfection

MC3T3-E1 was cultured in α-modified Eagle's medium (α-MEM) supplemented with 10% FBS. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The human mesenchymal stem cells (RCB2080; Riken BRC) were maintained using the MSCGM Mesenchymal Stem Cell Growth Medium BulletKit (Lonza). Cells were transiently transfected with mimic miR-145 (20 nM) or miR-34c (50 nM) using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. For osteoblastic differentiation, cells were cultured in osteogenic medium (Ob medium with 0.1 mg/mL ascorbic acid, 10 mM β-glycerophosphate) changed every 2 days. After 7 days, osteoblast differentiation was confirmed by measuring the of alkali phosphatase (ALP) activity and the expression of osteoblast marker genes as previously described [32]. ALP activity was measured using *p*-nitrophenyl phosphatase hexahydrate (Sigma). In brief, cells were lysed in 0.2% Triton-X solution. After sonication, the ALP activity in the cell lysates was determined and normalized to protein concentration. For von Kossa staining, MC3T3-E1 cells were cultured in Ob medium. After 21 days, cells were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and stained with 5% silver nitrate solution. After washing in distilled water, cells were incubated with 5% sodium thiosulfate solution. In vitro osteoclast differentiation was accomplished as previously described [33]. Briefly, bone marrow cells (3×10^5 cells per cm^2) from the femurs of six-to eight-week-old mice were cultured in α-MEM supplemented with FBS in the presence of human macrophage colony-stimulating factor (M-CSF, 10 ng/mL, R&D Systems) for 2 days and then differentiated into osteoclasts by exposure to human RANKL (50 ng/mL, Peprotech) and M-CSF for 3 days. Subsequently, the differentiation of osteoclasts was evaluated by TRAP staining. In brief, osteoclasts were fixed in 4% PFA in PBS. Fixed cells were permeabilized with acetone/ethanol and stained with Fast Red Violet LB Salt (Sigma) in TRAP solution [33]. To determine miR-145 expression, primary osteoblasts were isolated from calvaria of post-natal day 3 (P3) mice by digestion medium (0.5 mg/mL collagenase P and 0.05% trypsin in αMEM) and cultured in α-MEM supplemented with 10% FBS. After 3 days, the RNA was extracted from these cells.

2.3. Luciferase assay

Mouse Cbfb 3′-UTR regions were amplified by PCR and cloned into pCMV-luc reporter plasmid [22]. MiR-145 binding (5′-TCTAGAAACTGGAGGCGCGCCAACTGGACTGCAG-3′) or mutated oligonucleotides (5′-TCTAGAAGATCTCGGCGCGCCAGATCTCCTGCAG-3′) were cloned into pCMV-luc. HEK293 cells were plated in 24-well plates at a concentration of 1.3×10^5 cells/well. Cells were co-transfected with reporter plasmids (100 ng/well) and either the miR-145 expression vector (100 ng/well) or the control plasmid (100 ng/well) using Lipofectamine 2000 (Invitrogen). The phRL-tk plasmid (10 ng/well) was used as an internal control. We used the empty vector to equalize the amount of plasmid transfected in each experiment. After 48 h of transfection, firefly or *Renilla* luciferase activities were quantified using a dual-luciferase assay system (Promega).

2.4. Quantitative real-time PCR analysis

MiRNA and total RNA from tissues and cultured cells were extracted using the miRNeasy mini kit (Qiagen). RNAs were reverse transcribed using the TaqMan miRNA reverse transcription kit (Applied Biosystems) or the ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. Relative miRNA expression was determined by quantitative real-time PCR (qPCR) using miR-145- or miR-34c-specific TaqMan probes (Applied Biosystems). Osteoblast marker qPCR was performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). All qPCR reactions were performed using the Mx3000P real-time PCR system (Agilent Technologies). We used snoRNA202 (for miRNA) and GAPDH (for osteoblast marker gene) expression as internal controls. The following primers were used: *Alpl* S, 5′-ACACCTTGACTGTGGTTACTGCTGA-3′ and *Alpl* AS, 5′-CCTTGTAGCCAGGCCCGTTA-3′; *Bglap* S, 5′-TCTGACAAAGCCTTCATGTCCA-3′ and *Bglap* AS, CGGTCTTCAAGCCATACTGGTC-3′; *GAPDH* S, 5′-ACCCAGAAGACTGTGGATGG-3′ and *GAPDH* AS, 5′-CACATTGGGGGTAGGAACAC-3′.

2.5. Immunoblotting

Immunoblot analysis was performed according to a standard protocol. Cells were lysed in TNE buffer (10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing protease inhibitors. Lysates were subjected to SDS–polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Immunoblotting was performed using anti-Cbfb antibody (MBL; D127-3), anti-Runx2 antibody (MBL; M130-3), and β-actin antibody (Sigma; A2228).

2.6. Bone marrow ablation

Mice were anesthetized intraperitoneally with 400 mg/kg trichloroacetaldehyde monohydrate (Chloral Hydrate; Wako Pure Chemical). After removing the hair from both hind limbs, the bone marrow of both femora were ablated as described previously [32,34]. Briefly, bilateral longitudinal incisions were made on the knees of each mouse to expose the femoral condyle by dislocating the patella. A hole was made at the intercondylar notch of the femur using a dental drill. A 0.6 mm diameter Kirschner wire was inserted in the proximal end of the femur to confirm complete marrow ablation by radiography. Then, 20 μg of mimic miR-145 or the control was complexed with atelocollagen (Kohken) as described previously [35] and injected into the bone marrow. After injection, the dislocated patella was repositioned and the skin was sutured. Mice were euthanized at day seven after ablation and micro-CT analyses were performed. Animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University School of Medicine and conformed to the relevant guidelines and laws.

2.7. Statistical analysis

We performed statistical analysis using the Tukey–Kramer and Student's *t* test. Values were considered to be statistically significant at $P < 0.05$. All data are reported as the mean ± S.D.

3. Results

3.1. Identification of miRNA targeting Cbfb

First, we performed a comprehensive analysis of osteoblastic miRNAs using MC3T3-E1 osteoblastic cells. In parallel with this analysis, we also performed an miRNA database search to identify miRNAs that target Cbfb (Fig. 1A).

Out of the number of miRNAs that exhibited altered expression during osteoblast differentiation, we focused on miR-145 because it fulfilled two prerequisites of this study: it is expressed at substantial levels in osteoblasts and it targets Cbfb.

First, to confirm that Cbfb is a true target of miR-145, we performed a luciferase activity assay using a reporter plasmid in which the putative miR-145 binding sites of the Cbfb 3′UTR were cloned into the 3′UTR of the luciferase gene (Fig. 1B). In accordance with the in silico prediction, forced expression of miR-145 significantly decreased the luciferase activity, whereas a mutation in the miR-145 binding site of the Cbfb 3′UTR abrogated the response to miR-145 (Fig. 1C). Moreover, miR-145 decreased the luciferase activity of a construct harboring 1 kbp of the Cbfb 3′UTR fragment, which contains the aforementioned putative miR-145 binding site (Fig. 1D).

More importantly, the overexpression of miR-145 decreased Cbfb protein expression without overtly affecting Cbfb mRNA expression (Fig. 1E). These results indicate that miR-145 is a "bona fide" regulator of Cbfb.

3.2. Suppression of osteoblast differentiation by miR-145

Given that Cbfb is essential for proper osteoblast differentiation, we next studied whether miR-145 regulates osteoblast differentiation through the modulation of Cbfb expression. We first analyzed the expression profile of miR-145 throughout the whole body and observed that it is ubiquitously expressed in various tissues including bone, especially in osteoblasts (Fig. 2A). Moreover, miR-145 expression was induced during the time course of osteoblast differentiation, indicating that miR-145 physiologically affects osteoblast differentiation (Fig. 2B). Therefore, we next studied the effect of miR-145 on osteoblast differentiation by transfecting

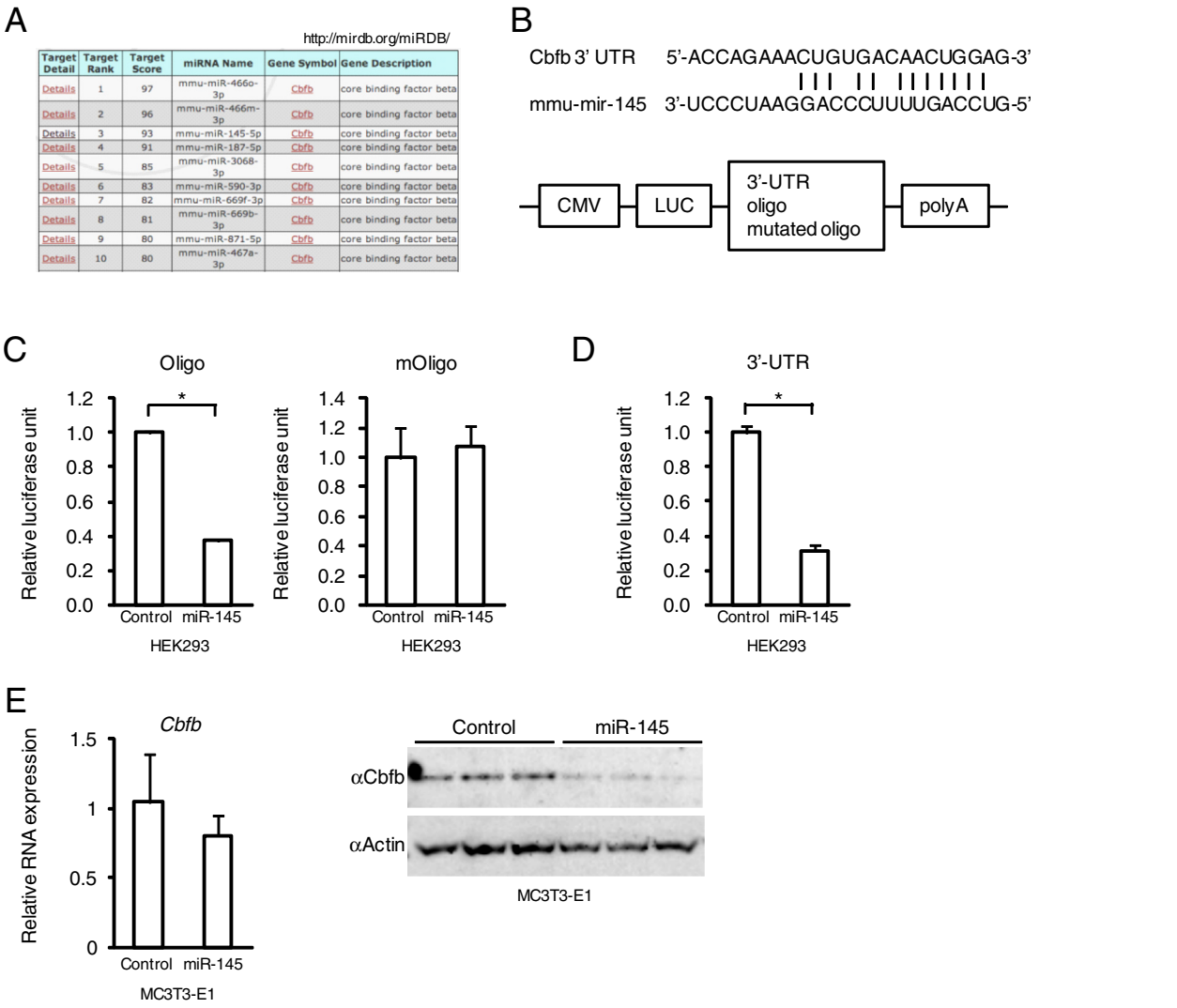

Fig. 1. Identification of Cbfb-targeting miRNA. (A) List of top 10 putative Cbfb-targeting miRNAs identified by in silico analysis (mirdb.org). (B) Schematic representation of the miR-145 target site in the 3′-UTR of Cbfb mRNA and the reporter construct. (C and D) Repression of Luc reporter activities mediated by the 3′-UTR of Cbfb mRNA. HEK293 cells were co-transfected with the reporter plasmid and miR-145 or the control. After 24 h, a luciferase assay was performed and normalized to *Renilla* expression. (E) MC3T3-E1 cells were transfected with mimic miR-145 or control. After 48 h, quantitative real-time PCR analysis of Cbfb mRNA expression (left) and Western blot analysis (right) were performed. The data are expressed as the mean ± S.D. of three independent experiments ($n = 3$). $^{*}P < 0.05$.

MC3T3-E1 or hMSC cells with miR-145. As expected, the overexpression of miR-145 inhibited osteoblast differentiation as shown by the decrease in the ALP activity and the expression of osteoblast markers such as *alpl* and *bglap* (Fig. 2C and D). Moreover, forced expression of a miR-145 mimic led to a significant decrease in matrix mineralization (Fig. 2E). To address whether the inhibition of osteoblast differentiation by miR-145 is due to Cbfb downregulation, we restored the expression of Cbfb in miR-145 transfected cells. Indeed, the decrease in ALP activity was rescued by the forced expression of Cbfb (Fig. 2F). In contrast to osteoblast differentiation, miR-145 did not affect osteoclast differentiation (Fig. 2G). These results suggest that miR-145 inhibits osteoblast differentiation through Cbfb expression.

3.3. miR-145 and miR-34c cooperatively inhibit osteoblast differentiation

Cbfb is required for Runx2-dependent transcription during osteoblast differentiation. Therefore, we hypothesized that miRNA targeting Runx2 and miR-145, which targets Cbfb, may also coordinately to regulate osteoblast differentiation. miR-34c inhibits osteoblast differentiation by regulating Runx2 expression [30].

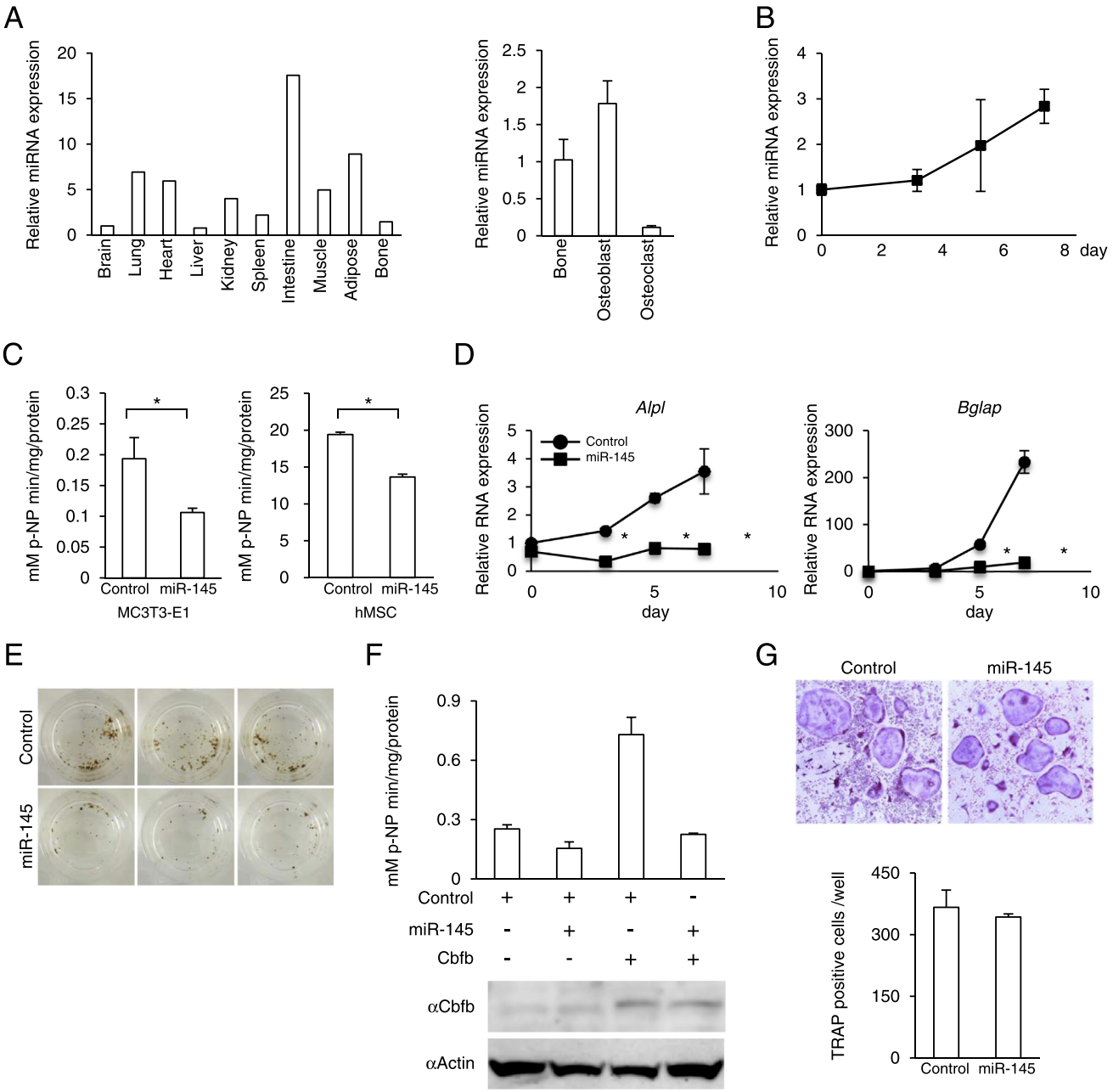

Fig. 2. Effect of miR-145 on osteoblast differentiation. (A) Expression pattern of miR-145 determined by real-time PCR. Total RNA was isolated from the indicated tissue, primary osteoblasts and osteoclasts. (B) Expression profiles of miR-145 during osteoblast differentiation. MC3T3-E1 cells were treated with differentiation medium for the indicated time. (C and D) Effect of miR-145 on osteoblast differentiation. Cells were transfected with mimic miR-145 or control. ALP activity (C) and osteoblast marker expression (D) were analyzed. (E) von Kossa staining of matrix mineralization on day 21. (F) Cbfb rescues the inhibitory effect of miR-145 on osteoblast differentiation. MC3T3-E1 cells were transfected with the indicated plasmids. After 7 days, the ALP activity was analyzed. (G) Effect of miR-145 on osteoclast differentiation. Primary osteoclasts were transfected with mimic miR-145 or control. Subsequently, TRAP-positive multi-nuclear cells were counted. The data are expressed as the mean ± S.D. of three independent experiments ($n = 3$). $^{*}P < 0.05$.

Similar to miR-145, the miR-34c expression was also induced during osteoblast differentiation, and transfection with miR-34c decreased ALP activity (Fig. 3A). The co-transfection of the miR-145 mimic and the -34c mimic inhibited ALP activity synergistically, albeit not significant, (Fig. 3B) compared with miR-145 mimic or miR-34c alone. MiR-145 and -34c decreased Cbfb or Runx2 protein expression, respectively (Fig. 3C). These results indicate that miR-145 and -34c cooperatively inhibit osteoblast differentiation through Cbfb and Runx2 protein expression.

3.4. miR-145 affects physiological bone formation in vivo

Next, to address the functional role of miR-145 in vivo, we ablated the bone marrow of mouse femurs and then treated them with miR-145. Treatment with mimic miR-145 reduced bone regeneration compared with mice treated with control scrambled miRNA (Fig. 4A and B). These results indicate that miR-145 also participates in the bone regeneration process in adult mice.

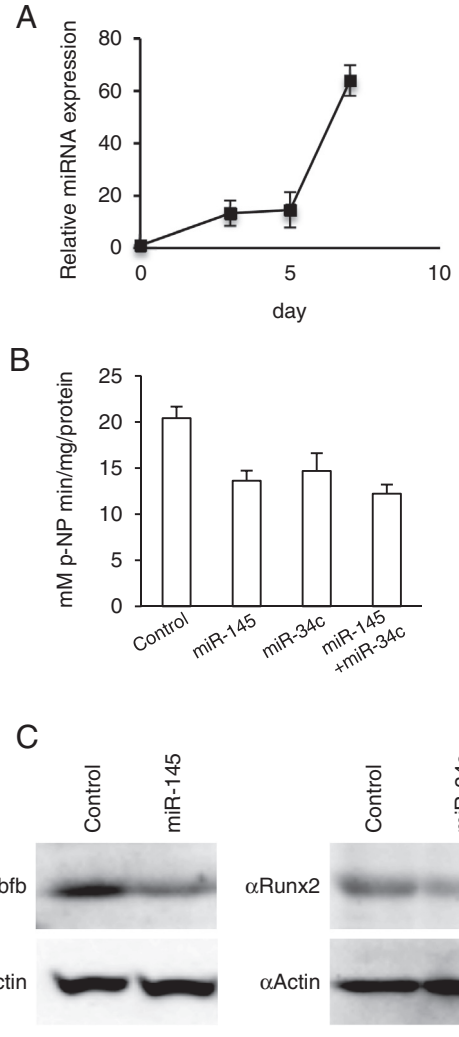

Fig. 3. miR-145 and miR-34c cooperatively inhibit osteoblast differentiation. (A) Expression profiles of miR-34c during osteoblast differentiation. MC3T3-E1 cells were treated with differentiation medium for the indicated amount of time. (B) Effects of miR-145 and miR-34c on osteoblast differentiation. MC3T3-E1 cells were transfected with mimic miR-145, miR-34c and control. After 7 days, ALP activity was analyzed. (C) MC3T3-E1 cells were transfected with mimic miR-145, miR-34c and control for 48 h. Western blot was used to detect the levels of Cbfb and Runx2 protein expression. The data are expressed as the mean ± S.D. of three independent experiments ($n = 3$).

4. Discussion

Bone homeostasis is strictly regulated by many physiological signals. In particular, osteoblasts play an important role in skeletal development and bone formation. Understanding the regulatory mechanism of osteoblast differentiation is important for the development of new therapeutic strategies to treat degenerative bone diseases such as osteoporosis. In this study, we investigated the miRNA-mediated regulation of Cbfb expression in osteoblast differentiation. We demonstrated that miR-145 directly regulates Cbfb expression. MiR-145 expression increased during osteoblast differentiation, and the ectopic expression of miR-145 suppressed osteoblast differentiation. The overexpression of Cbfb rescued the inhibitory effect of miR-145 in osteoblast differentiation. Finally, miR-145 affected the physiological process of bone regeneration in vivo.

Cbfb is a non-DNA-binding transcription factor that allosterically potentiates the DNA binding of Runx family proteins and plays an essential role in Runx2-dependent skeletal development. However, the mode of action of Cbfb remains unclear. Although Cbfb has been shown to stabilize Runx2 by inhibiting polyubiquitination-mediated proteasomal degradation [36], Cbfb

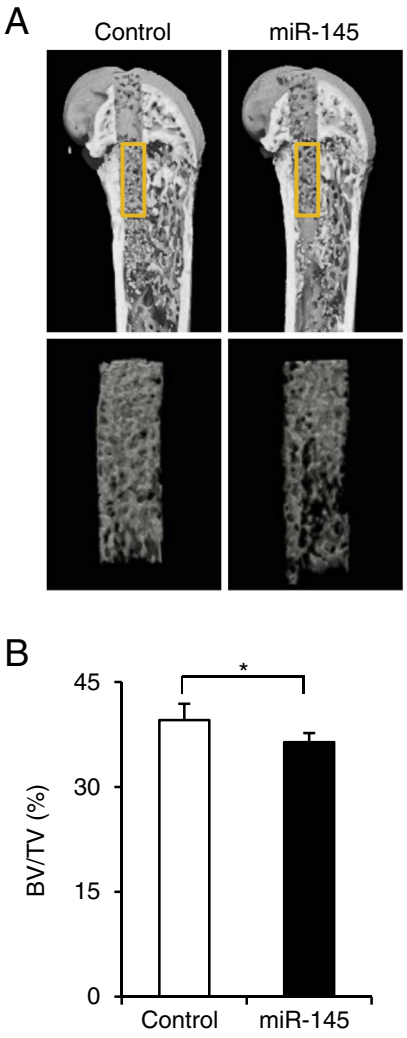

Fig. 4. miR-145 affects physiological bone formation in vivo (A) Representative micro-CT images of bone regeneration after femoral bone marrow ablation in mimic control and miR-145-treated and control-treated mice at 2 months. (B) Bone volume/tissue volume (BV/TV; %). Measurement areas were indicated in (A). The data are expressed as the mean ± S.D. ($n = 5$). $^{*}P < 0.05$.

does not interact with Runx2, at least in vitro [37]. Moreover, Cbfb haploinsufficiency does not elicit cleidocranial dysplasia [16]. Thus, Cbfb is important but not essential for osteoblast differentiation by itself, and it requires the presence of Runx2 to exert its effects on osteoblasts. Runx2 is a central regulator of osteoblast differentiation, and many miRNAs have been shown to affect osteoblast differentiation by modulating the Runx2 protein.

We identified that miR-145 directly regulates Cbfb expression by binding the putative 3′-UTR target site, resulting in the inhibition of Cbfb mRNA translation. We also showed that the overexpression of miR-145 inhibits osteoblast marker expression. These results suggest that miR-145 regulates runx2-dependent osteoblast differentiation through Cbfb. Indeed, the restoration of Cbfb expression was sufficient to rescue the abnormal osteoblast differentiation.

Interestingly, previous studies have shown that miR-145 regulates Osx protein expression, i.e., Osx is a target of miR-145 [38]. Osx is a zinc finger transcription factor that is essential for skeletal development and bone formation [39], and this gene is downstream of Runx2 in the transcriptional regulation of osteoblast differentiation. Thus, miR-145 act as a major regulator of osteoblast differentiation by affecting essential transcription factors, i.e., Cbfb and Osx. A mouse model lacking miR-145 would be useful to demonstrate the physiological importance of miR-145 for osteoblast differentiation in vivo.

Cbfb also forms a heterodimer with Runx1, and this complex is essential for hematopoiesis in the fetal liver. In addition, chromosomal translocation involving Cbfb is a major cause of hematopoietic diseases, such as inv (16) acute myeloid leukemia (AML). Indeed, both Cbfb- and Runx1-deficient mice develop impaired hematopoiesis [40,41]. We showed that miR-145 is ubiquitously expressed in many tissues including liver and bone, indicating that miR-145 may also be involved in the hematopoiesis and development of AML through Cbfb expression.

One microRNA targets a number of genes, and each target gene is regulated by many microRNAs. Thus, the relationship between an miRNAs and a target genes is a many-to-many correspondence, not a one-to-one correspondence. Recently, the miR-34 family has been reported to act as regulators of osteoblastogenesis. Osteoblast-specific deletion of miR-34 revealed that miR-34b and c play important roles in osteoblast proliferation by suppressing Cyclin D1, CDK4 and CDK6 and differentiation by repressing SATB2 [42]. These reports combined with our results suggest that miR-145 and -34c regulate osteoblastogenesis through a number of target genes.

In this study, we demonstrated that different microRNAs cooperatively regulate a common signaling pathway by affecting one specific transcription factor complex, i.e., Runx2-Cbfb. Indeed, transfecting multiple miRNAs targeting Runx2-Cbfb synergistically decreased osteoblast differentiation.

Thus, beyond regulating the gene transcription/translation by many-to-many correspondence, multiple microRNAs cooperatively regulate one physiological event, i.e., osteoblast differentiation, by affecting the same transcription factor complex.

Interestingly, recent reports showed that Cbfb stabilizes the Runx2 protein and thus stimulates osteoblast differentiation [36]. Together with our results showing that miR-145 regulates osteoblast differentiation through Cbfb, these results clearly demonstrate the importance of Cbfb in osteoblast differentiation.

Until now, the molecular mechanism of the regulation of miR-145 expression during osteoblast differentiation has remained unclear. Previous reports showed that the promoter activity of miR-145 was repressed by OCT4 in human embryonic stem (ES) cells [43]. OCT4 is a fundamental transcription factor that regulates the self-renewal and differentiation of ES cells and is expressed in bone marrow-derived mesenchymal stem cells [44]. Therefore, Oct4 is a plausible candidate miR-145 regulatory factor. Further study will be required to elucidate the detailed regulatory mechanism of miR-145 expression.

In summary, we have demonstrated that miR-145 is a novel regulator of Cbfb expression and that it acts as a global regulator of osteoblast differentiation. Our findings suggested that the pharmacological inhibition of miR-145 could lead to a therapeutic approach to treat degenerative bone diseases, such as osteoporosis.

Acknowledgements

We thank K. Katagiri, T. Hara, S. Ohba and M. Iwata for technical assistance. This work was supported in part by a grant-in-aid for scientific research on innovative areas (“Functional machinery for non-coding RNAs”) to T.F., a grant-in-aid for scientific research to T.F. and S.T. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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